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CELL CYCLE ARREST AS A BASIS OF ENHANCEMENT OF 1-β-D-ARABINOFURANOSYLCYTOSINE ANABOLISM IN HUMAN LYMPHOBLASTOID RPMI 6410 CELLS CULTURED WITH 3-DEAZAURIDINE*

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Abstract—The antiproliferative effect of 1-β-D-arabinofuranosylcytosine (araC) on RPMI 6410 cells in culture was potentiated in the presence of 3-deazauridine (DU). When the culture medium contained DU, proliferation ceased and cells did not progress in the replication cycle beyond early S phase. When cells from such DU-treated cultures were transferred to DU-free medium containing Cyd, a rapid, partially synchronous resumption of DNA synthesis occurred. Under these circumstances, the activity of dCyd kinase, assessed using araC as substrate, was enhanced in unfractionated extracts of DU-treated cells relative to extracts of untreated cells. It is suggested that the presence of DU in culture medium stopped the progression of cells through the replication cycle, arresting them in an araC-sensitive portion of S phase, and that the enhanced ability of DU-treated cells to anabolize dCyd and araC derived, at least in part, from the accumulation of cells in S phase, a portion of the cell cycle in which dCyd kinase is elevated.

DU,¶ a uridine analogue that inhibits proliferation of microbial cells and neoplastic cells in culture [1, 2], is converted in animal cells and tissues to 5′-phosphate derivatives, including DU 5′-triphosphate [3–5]. Earlier reports from this laboratory showed that resistance to DU in RPMI 6410 cells was associated with deficiency in uridine kinase [2, 6], indicating that DU is a substrate for that enzyme, as others have shown [7, 8], and that conversion to phosphorylated metabolites is essential to the expression of DU cytotoxicity. DU is not incorporated into RNA or DNA, and formation of 2′-deoxy derivatives of DU was not in evidence in murine neoplastic cells [3]. Inhibition of CTP synthetase by DU 5′-triphosphate [4] is the evident basis of the pronounced depletion of cellular cytosine nucleotides observed when cells are treated with DU [9, 10]. A consequence of the DU-induced perturbation of nucleotide metabolism is enhancement (in the presence of DU) of the anabolism (1) of dCyd [11] and araC [2, 10, 11] in cultured cells, and (2) of araC in leukemia L1210 cells in mice [12]. The latter effect suggested that DU might modify the toxicity of araC, and we have reported that the toxicities of AraC and DU toward mice [2, 13], HeLa cells [2], and RPMI 6410 cells [2, 10] were enhanced synergistically by the joint administration or presence of these agents.

The present study tested the idea that the apparent enhancement of araC anabolism in DU-treated cells was due, in part, to enhanced dCyd kinase activity as a result of DU-induced arrest of progression through the cellular replication cycle. Cellular deoxycytidine kinase activity changes during the replication cycle, being lowest during G_1 phase, increasing several-fold with progression into S phase, and declining thereafter during G_2 phase and mitosis [14, 15].

DU-induced perturbations of cellular nucleotide metabolism may also contribute to the enhancement of araC anabolism by DU. For example, the DU-induced reduction of cellular concentrations of dCTP, an allosteric inhibitor of dCyd kinase [16, 17], could relieve feedback inhibition of that enzyme, as suggested by others [11]. Further, it is possible that the intracellular presence of DU metabolities may reduce the catabolism of araC metabolites; in this respect, the 5′-diphosphate of DU was shown recently to inhibit Cyd and dCMP deaminase activity isolated from human granulocytes [18].

MATERIALS AND METHODS

[Methyl-^{3}H]dThd and [5,6-^{3}H]araC were purchased from Moravek Biochemicals, Brea, CA, U.S.A. DU was provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A. 6-Aminocaproic acid was purchased from the Aldrich Chemical Co., Milwaukee, WI, U.S.A. and other chemicals from the Sigma Chemical Co., St. Louis, MO, U.S.A. Cell culture materials were obtained from GIBCO Canada Ltd.,

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¶ Abbreviations: DU (3-deazauridine), 4-hydroxy-1-β-D-ribopentofuranosyl-2-pyridone; and araC, 1-β-D-arabinofuranosylcytosine.

Burlington, Ontario, Canada. Sepharose 4B was purchased from Pharmacia (Canada) Ltd., Dorval, Quebec, Canada.

Static cultures of RPMI 6410 cells, a human lymphoblastoid B-cell line [19, 20], were maintained at 37° in an atmosphere of 5% CO_2 in air in RPMI 1640 medium supplemented with 10% fetal calf serum. Cell concentrations, kept below 5×10^5 cells/ml, doubled every 17 to 19 hr. Dialyzed fetal calf serum was used in all experiments in which cells were treated with drugs.

In experiments concerned with cell cycle arrest by DU, cells were incubated in growth medium containing 10 μM DU for 16 hr, collected by centrifugation (150 *g* for 5 min), and resuspended in fresh, warmed medium with or without additives. DU-induced changes in the frequency distribution of cellular DNA content in cell populations so treated were appraised by flow microfluorimetry.* At intervals following transfer of cells to fresh medium, cell concentration, mitotic indices and incorporation of dThd into acid-insoluble material were measured.

In assaying the incorporation of dThd into acid-insoluble material, mixtures (3 to 6×10^5 cells in 1.1 ml of growth medium containing 11 nM [methyl-^{3}H]dThd (1 μCi)) were incubated at 37° for 10 min, an interval during which incorporation was linear with time. After addition of 10 ml of cold 5% trichloroacetic acid, the precipitated material was collected on nitrocellulose filters which were washed and assayed for ^{3}H after combustion (Packard model 306 Sample Oxidizer) by liquid scintillation counting.

For mitotic index determinations, samples of about 10^5 cells were centrifuged onto slides that were exposed to Wright's stain (250 mg stain in 100 ml methanol) for 4 min, then to 5 mM KH_2PO_4 (pH 6.8) for 8 min, and rinsed with water. At least 2000 cells were examined in each determination of mitotic index.

Cell extracts for assay of araC phosphorylating activity (dCyd kinase) were prepared as follows. Culture samples containing 10^8 cells were cooled to 4° and the cells were pelleted, washed with cold 0.15 M NaCl and finally resuspended in 1.0 ml of cold extraction buffer [0.01 M Tris (pH 7.5), 10% (v/v) glycerol, 0.15 M NaCl, 2 mM dithioerythritol and 20 mM 6-aminocaproic acid, Ref. 16]. Cell suspensions were than rapidly frozen and thawed four times and homogenized at 4° with thirty strokes of a Dounce homogenizer. The homogenates were centrifuged (10,000 *g* at 4° for 10 min) and supernatant fractions were assayed for dCyd kinase activity using araC as the substrate. Kinase assay mixtures [16] contained, in a final volume of 0.05 ml, 0.1 M Tris–HCl (pH 7.5), 6 mM ATP, 6 mM $MgCl_2$, 0.1 mg ovalbumin, 6 mM creatine phosphate, 8 mM NaF, 2 mM dithioerythritol, 1 unit of creatine kinase, cell extract, and various concentrations of [5,6-^{3}H]araC. Assay mixtures were incubated at 37° for 15 min; rates of product formation were constant during this interval and were proportional to protein concentration. To end assay intervals, 25-μl samples of the assay mixtures were placed on squares (2.5 cm^2) of DEAE cellulose paper (Whatman DE-81 paper, Reeve Angel, Clifton, NJ, U.S.A.) that were immersed individually, each in a 10-ml portion of 0.1 mM ammonium formate (pH 3.4). Each square was then rinsed three times with 10 ml of the latter, once with 10 ml water and once with 10 ml of 95% ethanol, and were assayed for ^{3}H by the combustion–liquid scintillation procedure. The response in assay mixtures containing heated extract (100°, 3 min) was 50–60 cpm above background. The 10,000 *g* sediments (see above) did not contain measurable araC phosphorylating activity. Protein was determined by the method of Hartree [22].

The cell extracts (see above) were subjected to affinity chromatography on dThd-linked Sepharose 4B gel [16]†. The dThd content of the gel was 9.2 μmoles/g of dried gel.

RESULTS

Our earlier reports [2, 10, 12] showed that (1) growth inhibitory effects of araC and DU toward cultured RPMI 6410 cells‡ and HeLa cells were enhanced synergistically when these agents were present together, and (2) the anabolism of araC was enhanced in RPMI 6410 cells in the presence of DU and in leukemia L1210 cells pretreated *in vivo* with DU. Experiments described here represent an attempt to perceive in RPMI 6410 cells a biochemical basis for the araC–DU synergism in growth inhibition.

Figure 1 illustrates the influence of DU exposure on the frequency distribution of the DNA content in a population of RPMI 6410 cells. As the DU-exposure interval increased, the proportion of cells in the G_2 + M phases of the cell cycle decreased, while the G_1 peak of the distribution broadened and shifted toward higher DNA content. The results suggest that, following exposure to DU for 16 hr or longer, cells accumulated at a stage of S phase in which the DNA content was somewhat greater than that of G_1 phase cells.

Incubation of RPMI 6410 cells for 16 hr in medium containing 10 μM DU blocked cellular DNA synthesis, as measured by incorporation of labeled [^{3}H]dThd into acid-insoluble material during a 10-min assay interval. When DU-treated cells were transferred to fresh DU-free culture medium supplemented with 2 μM Cyd, cells regained the ability to incorporate [^{3}H]dThd into acid-insoluble material (Fig. 2A), and the rate of this apparent DNA synthesis was at a maximum about 2 hr after the change of medium. A second broader peak of cellular DNA synthesizing activity occurred between 16 and 25 hr after the medium change (data not shown). Following transfer to DU-free medium lacking Cyd, the incorporation of dThd began after a lag of 1–2 hr, which might represent the interval necessary for replenishment of cellular Cyd and dCyd phosphates. At concentrations between 2 and 10 μM, Cyd was

* Flow microfluorimetric analyses [21] were performed in the laboratory of Dr. L. W. Brox, to whom we are grateful.

† We are grateful to Dr. T. P. Lynch for preparation of the gel.

‡ The proliferation rate (cell number doublings in 48 hr) of RPMI 6410 cells was halved in medium containing 4.8 μM DU or 0.3 μM araC.

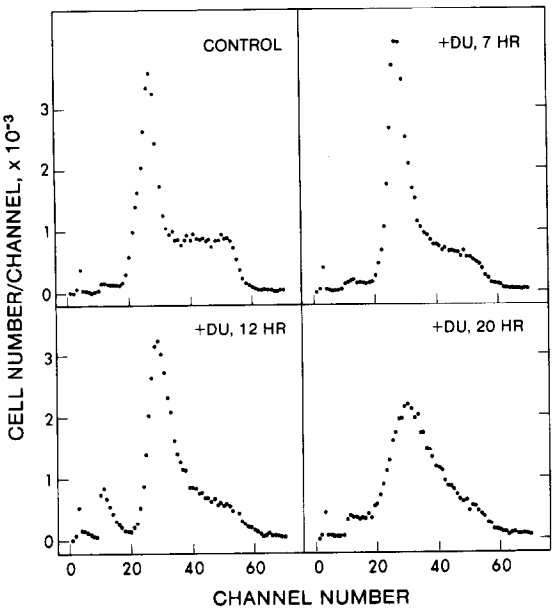

Fig. 1. Distribution of DNA content in RPMI 6410 cells. After exposure to 10 μM DU for the times indicated, the frequency distribution of cellular DNA content was determined by flow microfluorimetry.

more effective than dCyd in restoring DNA synthesizing activity in DU-arrested cells (data not shown).

Figure 2B shows that restoration of DNA synthesis in DU-treated cells was followed by cell division, as indicated by increases in cell numbers and corresponding increases in mitotic indices. The shoulder on the cell concentration profile (Fig. 2B) was also evident in the mitotic index data; both were apparent in two similar experiments. This result may mean cells were arrested by DU at two different loci in the cell cycle.

The activity of cellular dCyd kinase in animal cells changes markedly with progression through the replication cycle, reaching higher levels during S phase [14, 15]. The experiment of Fig. 3 explored the relationships between araC concentration and rates at which araC phosphates were formed by extracts from untreated and DU-treated cells. It is seen that reciprocal plots were linear and that the values of the kinetic constants K_m and V_{max} for "araC kinase" activity extracted from DU-treated cells were apparently greater than the corresponding parameters for the kinase activity from untreated cells. Table 1 summarizes the kinetic parameters (obtained from reciprocal plots as in Fig. 3) for araC kinase activity of cells cultured in the presence or absence of DU. The products of the araC kinase reaction formed in assay mixtures (see Materials and Methods) were determined by t.l.c. [10]. When extracts from untreated cells were assayed in this way, the relative proportions of the 5′-mono-, di- and triphosphate esters of araC formed were 36:25:39 after 15 min of incubation; when extracts from DU-treated cells were assayed, the relative proportions of the three phosphate esters formed were 52:23:25. About 2 per cent of the [5,6-^{3}H]araC substrate was converted to araU during the 15-min incubation interval with extract from untreated or DU-treated cells.

The presence of DU (10 μM) in araC kinase assay mixtures did not influence the kinase activity determinations. When extracts from DU-treated or untreated cells were dialyzed against several changes

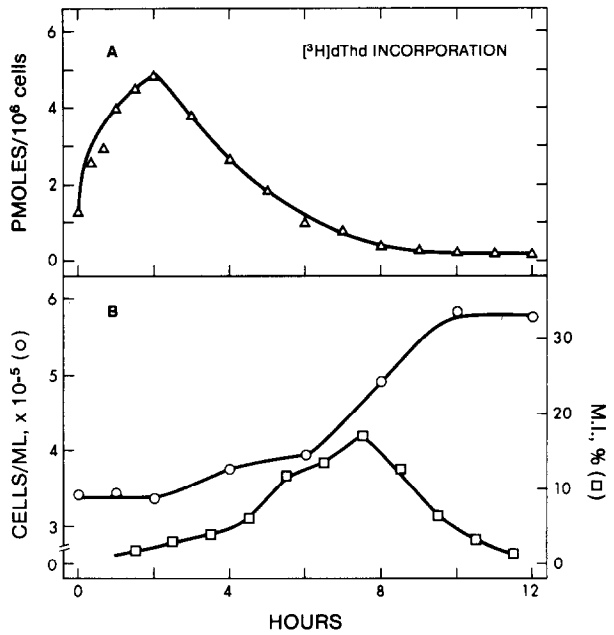

Fig. 2. Synchronization of RPMI 6410 cells by DU. After exposure to 10 μM DU for 16 hr, cells were resuspended (time zero) in DU-free growth medium supplemented with 2 μM Cyd and incubated at 37°. Measured at the intervals indicated were: Panel A, incorporation of dThd into acid-insoluble material; and Panel B, mitotic index and cell number. Incorporation of dThd into acid-insoluble material of untreated cell cultures was 0.8 to 1.0 pmole per 10^6 cells.

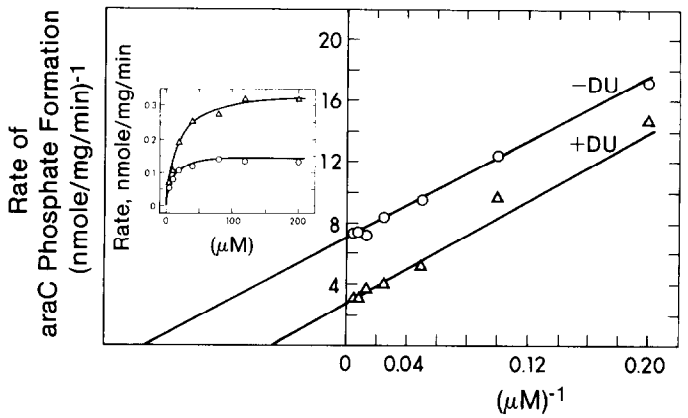

Fig. 3. Influence of araC concentration on the rate of araC phosphorylation by cell extracts. Rates of araC phosphate formation per mg protein in extracts of untreated and DU-treated (10 μM, 12 hr) RPMI 6410 cells were determined during 15-min incubations with graded [5,6-^{3}H]araC concentrations (1.72×10^5 cpm per assay). Reaction mixtures contained 29.3 or 15.6 μg protein from extracts of untreated or DU-treated cells respectively; each point represents the mean of triplicate determinations. Lines were fitted to the points by the methods of least squares.

of extraction buffer, both K_m and V_{max} values for the araC kinase activity from DU-treated cells remained elevated relative to the corresponding values obtained with extracts from untreated cells. When extracts from untreated and DU-treated cells were present together in the same assay mixtures, the resulting araC kinase activity approximated the sum of the activities of the individual extracts (data not shown). Together these observations demonstrate that the enhanced activity of araC kinase in extracts from DU-treated cells was not attributable to (1) the presence of dissociable activators, (2) the presence of feedback inhibitors (e.g. dCTP) in extracts from control cells, or (3) a direct influence of DU or its anabolites present in extracts from DU-treated cells.

AraC kinase in unfractionated extracts from DU-treated or untreated RPMI 6410 cells was partly purified by affinity chromatography on columns of Sepharose 4B containing covalently bound dThd [16]. Elution was accomplished as described in Fig. 4; fractions 22–25 contained most of the araC phosphorylating activity in both enzyme preparations. In that experiment, the extract from DU-treated cells yielded 1.8 times more araC phosphorylating activity in these fractions than did extract from untreated cells containing an equivalent amount of protein. The single peaks of activity observed in Fig. 4 probably represent the cytoplasmic isozyme of dCyd kinase since araC does not serve as substrate for the mitochondrial isozyme extracted from human leukemic lymphocytes [16].

DISCUSSION

When proliferating RPMI 6410 cells are placed in growth medium containing 10 μM DU, cells become depleted of cytosine nucleotides and proliferation ceases. It is reasonable to suppose that these are sequelae of the inhibition of CTP synthetase by DU 5′-triphosphate. The present study suggests that, in the presence of DU, cells do not progress through the replication cycle beyond an early portion of S phase and that cells in this state accumulate in DU-treated cultures. These conclusions derive from

Table 1. Apparent kinetic constants for araC phosphorylation by extracts from RPMI 6410 cells*

		Apparent kinetic constants	
Expt.	Treatment	K_m (μM)	V_{max} [nmoles·(mg protein)$^{-1}$·min^{-1}] × 10^2
1	None†	7.5	14
	DU†	22.7	37
2	None	6.1	21
	DU	19.3	46
3	None	7.3	20
	DU	15.5	53
4	None	7.5	16
	DU	20.2	45

* AraC kinase activity of extracts prepared from untreated or DU-treated (10μM, 12 hr) cells was determined at graded concentrations of araC as described in Fig. 3; kinetic constants were obtained from lines fitted by the least squares method to reciprocals of the rate and concentration data.

† Experiment of Fig. 3.

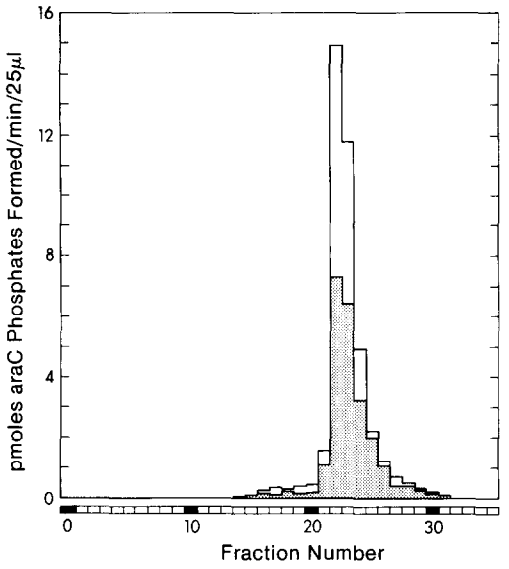

Fig. 4. Affinity chromatography of araC kinase. Extracts (1 ml, 4.2 mg protein) from untreated (shaded portion) and DU-treated (10 μM, 12 hr) RPMI 6410 cells were applied to 0.5 × 2 cm columns of dThd-linked Sepharose 4B gel previously equilibrated with 0.01 M Tris–HCl buffer (pH 7.5) containing 10% glycerol and 5 mM dithioerythritol. Elution (1 ml fractions) was accomplished at 4° with the following solutions containing glycerol and dithioerythritol as above: fractions 2–11, equilibration buffer; fractions 12–17, 0.2 M Tris–HCl buffer (pH 7.5); fractions 18–20, the latter containing 100 μM dThd; fractions 21–23, 0.4 M Tris–HCl buffer (pH 7.5); fractions 24–28, the latter containing 300 μM dThd; and fractions 29–33, 0.4 M Tris–HCl buffer (pH 7.5) containing 600 μM dThd. Immediately after collection, ovalbumin (0.2 mg) was added to each fraction; 25-μl samples of each fraction were then assayed for dCyd kinase activity. Reaction volumes were 100 μl and contained 40 μM [5,6-^{3}H]araC as substrate. A confirmatory experiment yielded similar results.

observations that (1) transfer of DU-treated cells to DU-free growth medium containing Cyd resulted in a rapid, partially synchronous resumption of cellular DNA synthesis followed by cell division, and (2) the araC kinase activity of DU-treated cells was higher than that of untreated, proliferating cells, indicating an S phase state. The data of Fig. 2 suggest that a minor portion of the DU-treated cells was arrested later in the replication cycle, since mitotic activity was evident 2–3 hr after DU-treated cells were transferred to fresh medium.

The experiments reported here suggest that synchronization of cell populations by DU may be the basis of the potentiation in toxicity toward cultured cells [2, 10] and mice [2, 13] that results from the combination of DU and araC. These results indicate that arrest by DU during the progression of cells through the replication cycle places cells in a state of sensitivity to araC. S phase sensitivity of cultured cells to araC has been demonstrated [23, 24]. These considerations indicate that toxicity of DU–araC combinations toward proliferating cells would be related to the timing and sequence of exposure to these agents. We have reported [13] that lethality toward mice of treatment protocols with DU–araC combinations was markedly dependent upon the sequence of administration of these agents and the time interval between the injection of each. Treatment protocols in which DU was administered 3–6 hr before araC had the highest lethality and produced severe injury to the mucosa of the small bowel. The clear dependency of this tissue injury on sequence and schedule of administration of the two agents may be related to arrest of cycling cells in the susceptible tissue in an araC-sensitive state.

The DU-induced enhancement of araC and dCyd anabolism observed with cultured cells [2, 10, 11] and leukemia L1210 cells in mice [12] may derive from a partial accumulation of these cells in an S phase state in which dCyd kinase activity is elevated. As suggested previously [11], the DU-induced depletion of dCTP, an allosteric inhibitor of dCyd kinase [16, 17], would presumably tend to maximize rates of intracellular trapping (through phosphorylation) of influent dCyd and araC molecules. The enhancement by DU of dCyd and araC uptake by cultured cells has been demonstrated with short-term (minutes) assay procedures [10, 11].

The present results indicate that DU-treated cells had a higher content of extractable araC phosphorylating activity than did untreated cells and that the activity from both co-chromatographed on a dThd–Sepharose gel system capable of separating cytoplasmic and mitochondrial forms of dCyd kinase [16], suggesting (but not proving) that only one species of dCyd kinase was present. However, K_m and V_{max} values for the araC phosphorylating activity from DU-treated cells were increased relative to those obtained with extracts from untreated cells (Table 1, Fig. 3). Incubation of RPMI 6410 cells with hydroxyurea for 4–12 hr under culture conditions also increased the cellular content of extractable araC phosphorylating activity (data not shown).

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